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SYNTHESIS AND ANTIPLATELET ACTIVITIES OF SOME DERIVATIVES OF *p*-COUMARIC ACID

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Abstract

The aim of this research is the synthesis of *p*-coumaric acid (CA) derivatives and the in vivo examination of analgesic and antiplatelet activities. CA was reacted with dimethyl sulphate to obtain DMC, followed by base hydrolysis to obtain MCA, then catalytic hydrogenation using the Raney-Ni catalyst, yielding MPPA compound. In addition, CA was reacted through a Fisher esterification reaction, producing MC. All reactions except catalytic hydrogenation were conducted by utilizing microwave irradiation from a household microwave oven. The antiplatelet tests using slide clotting time showed that the results of CA and four compound derivatives have antiplatelet activity higher than the negative control. DMC, MCA, and MC groups clotting times showed significant improvements compared to CA. The potential of antiplatelet activity of DMC and MCA are not significantly different ($p < 0.05$) compared to aspirin as the positive control. The antiplatelet activities of CA and its derivatives are related to the lipophilic nature of the compound (Log P) and its affinity (RS) with the selected target molecule, i.e COX-1 enzyme (PDB ID 1CQE).

Keywords: *p*-coumaric acid, antiplatelet, COX-1, clotting time, catalytic hydrogenation, hydrolysis

1. Introduction

One of the leading causes of death from cardiovascular disease is thromboembolism. The accumulation of platelet formation plays an important role in the pathogenesis of thromboembolic disorders (1). Many endogenous compounds, e.g. thromboxane A2, thrombin, von Willebrand factor, and ADP that increase platelet aggregation on different routes. Platelets maintain a balance between anti-aggregator and pro-aggregator behavior under normal physiological conditions (2). The main treatment for this disease is antithrombotic, including antiplatelet. Aspirin is a widely used antiplatelet with the inhibiting mechanism of cyclooxygenase-1 (COX-1) enzyme (3). However, about 15-25% of patients are known to be resistant to aspirin. In addition, there are side effects in the form of bleeding and neutropenia (4;5).

One of the derivatives of the cinnamate compound, namely *p*-coumaric acid, [(E)-3-(4-hydroxyphenyl)acrylic acid] (CA) has been reported to have antiplatelet activity (6) (7). Zhao et al. (2016) reported that CA acts as an anti-inflammatory agent by reducing the expression of inducible Nitric Oxide Synthase (iNOS), COX-2, IL-1β, and TNF-α at mRNA and/or protein levels in RAW 264.7 cells stimulated by LPS. Molecule CA also decreases cytokine levels to reduce MAPK pathway activation, NF-κB, and decreases immune response (8).

One of the factors affecting antiplatelet activity is the drugs ability to penetrate cell membranes or lipophilicity of the compounds (7). To improve the lipophilicity, methylation reaction were carried out with dimethyl sulphate/base and Fisher esterification to modify carboxylic and OH-phenolic moieties so increasing the value of log P of CA derivatives. The hydrogenation of vinyl double bond will also be performed to determine the effect of double bond on both analgesic and antiplatelet activities.

The antiplatelet test was implemented by the blood clotting time slide method. The prediction of antiplatelet mechanisms was studied in the *in silico* study with COX-1

enzyme (9). The *in silico* strudy results were compared in terms of Rerank Score (RS) with aspirin.

2. Experimental

2.1. Materials

p-coumaric acid (CA) was bought from Aldrich; dimethyl sulphate, KOH, acetone, chloroform, potassium carbonate, HCl, H_2SO_4, NaOH, ether, ethanol, methanol, Raney-Ni were purchased from Merck. Derivatives of CA (i.e. DMC, MCA, MC, MPPA) were synthesized with several reactions (Fig. 1).

2.2. Methods

The synthesis reaction was monitored with TLC using UV lamp on λ254 nm to spot detection. Melting points were measured by Fischer-John melting point apparatus without correction. UV spectra were obtained by Shimadzu HP 8452 UV-vis spectrophotometer. IR spectra were performed using a Jasco FT-IR 5300 spectrophotometer. The ^1H and ^{13}C-NMR spectra were obtained from the JEOL JNM-ECS 400 instrument (^1H-NMR: 400 MHz, ^{13}C-NMR: 100 MHz) using $CDCl_3$ as a solvent. Physicochemical properties (log P, MR, E_{total}) were obtained by Chem Bio Ultra program.

2.2.1. (*E*)-methyl 3-(4-methoxyphenyl)acrylate (DMC)

p-coumaric acid (500 mg, 3.05 mmol) was dissolved in 10 ml of acetone. Potassium carbonate (1.25 g; 9.6 mmol) and dimethyl sulphate (2 ml; 21.9 mmol) were added into the mixture. The resulting mixture were irradiated in a microwave oven at 70 Watts power. The crude product was poured into an Erlenmeyer flask containing distilled water and stirred at room temperature, after which it was washed several times and then recrystallized with the appropriate solvent.

2.2.2. (*E*) -3- (4-methoxyphenyl)acrylic acid (MCA)

DMC (500mg; 2.60 mmol) was dissolved in a 20 ml of 5% KOH/ethanol solution. The mixture was irradiated into a microwave oven at 70 Watt power until the reaction is complete. Thereafter, the resulting mixture was acidified with HCl to yield sediment of *p*-methoxycinnamic acid (MCA). This crude product was purified by recrystallization using an appropriate solvent.

2.2.3. (*E*) -methyl 3- (4-hydroxyphenyl)acrylate (MC)

p-coumaric acid (500 mg; 3.05 mmol) was dissolved in a 5 mL of methanol, then 2 drops of concentrated H_2SO_4 were slowly added into the mixture. The mixture was introduced into a microwave oven and irradiated at 200 Watt power until the reaction was completed. The excess of methanol was evaporated, and then the residue was neutralized with a 5% potassium carbonate solution. The crude product was washed with cold water and then purified by recrystallization using an appropriate solvent.

2.2.4. 3-(4-methoxyphenyl)propanoic acid (MPPA)

MCA (500mg; 2.77 mmol) was dissolved in a 5% 25 ml NaOH solution (30 mmol). The mixture was heated until the MCA was dissolved and then cooled into room temperature again. A catalytic compound of Raney-Ni (565 mg; 5 mmol) was added to the mixture and stirred at room temperature for one hour. When the rate of gas release decreased, the

mixture was heated until the entire hydrogen g was exhausted (if the volume of the mixture was reduced during heating, sufficient water was added to the initial volume) then the mixture was filtered. The black precipitate of Ni residue on the filter paper was washed with 2 x 5 ml of 5% hot NaOH solution, followed by 2 x 5 ml of hot water. The filtrate was collected and cooled at room temperature. Then, 5 ml of concentrate HCl was dripped slowly and the temperature was maintained between 80-85ºC. The precipitate was extracted using 3 × 10 ml ether. The ether phase was evaporated and the forming crystals were dissolved in acetone to separate from the remaining Al mixed with the crystals. The acetone phase was evaporated, then white MPPA precipitate was obtained. This crude product was purified by recrystallization using an appropriate solvent (10).

2.2.5. Clotting Time Assay

White male adult mice in good health of age 8-12 weeks and 20-22 weight were adapted and fed for one week and then randomly divided into 5 groups, each consisting of 6 mice. Each mice was fed with their usual feed every day and were given drink of water *ad libitum*. All mice were tested for blood clotting time (day 0) and were put into the mounting. The blood clotting time was calculated by putting mice on an observation desk. The mice's tail was cleaned by 70% alcohol and then pierced with a surgical knife as far as 2 cm from the tail edge for a 2 mm puncture depth. The dripping blood were then dripped into the object glass and observed for every 15 seconds to determine the onset of fibrin formation. Afterwards, the scarred mice were treated using betadine solution according to their groups. Low dose of ASP (80 mg) was used as a positive control and 0,5% CMC-Na solution was used as negative control, both administered orally. The test solutions were given orally with the same dose of aspirin for 7 days. On the 8th day, blood clotting time on the test animals were analyzed according to the procedure outlined above (11). Antiplatelet assay was approved by the Ethical Commision of Airlangga University.

2.2.6. *In silico* study

The *in silico* study was performed on the crystal structure of the enzyme COX-1 (PDB ID 1CQE) with 2,90 Å resolution, which was downloaded from the RCSB Protein Data Bank (www.rcsb.org), in the form of binding ligand crystal COX-1 with FLP_1650 [A]. The complex 1CQE-FLP_1650 [A] crystal structure downloaded to the active site and determined for its binding sites. The 3D molecular structure of test compounds were imported into the active site and placed in a cavity in accordance with FLP_1650 [A]. The *in silico* assay was conducted into an appropriate cavity of the SE algorithm using MolDock with the maximum of 1500 iterations. The affinity of the ligand was determined and the score expressed as Rerank Score (RS). The complex enzyme ligand with pose with the highest score showed the best interaction. The best docking result must fulfill the requirements, namely the lowest energy and the position of the molecule that is in the same bond with FLP_1650 [A], observed visually. Observation of enzyme-ligand interactions included hydrogen bonding, steric interactions (Van der Waals), as well as electrostatic performed to pose with the highest score of RS (10).

3. Results and Discussion

The structure modification of the *p*-coumaric (CA) acid used the Fisher esterification reaction, alkylation, and catalytic dehydrogenation mentioned above, yielded the desired

compounds, namely DMC, MCA, MC, and MPPA (Fig. 1). The comprehensive physicochemical and spectral data of each compound that were obtained is as follows.

3.1. (*E*)-methyl 3-(4-methoxyphenyl)acrylate (DMC)

Obtained in 75% yield as white crystal; m.p. 88ºC. UV : λ_{max} (EtOH) 228 dan 310 nm. Spectrum IR (KBr; υ cm^{-1}): 2948, 1717, 1637, 1603, 1513, 1288, 1175, 823. Spectrum ^{1}H-NMR (DMSO-d6, δ ppm): 7.63 (2H,d, *J*=8.4 Hz), 7.57 (1H, d, *J* = 15.8 Hz), 6.93 (2H, d, *J* = 8.4 Hz) 6.44 (1H, d, *J* = 15.8 Hz), 3.76 ppm (3H, s), 3.66 (3H, s). Spectrum ^{13}C-NMR (DMSO-d6, δ ppm): 167.5, 161.7, 144.9, 130.7, 130.7, 127.1, 115.6, 114.9, 114.9, 55.9, 51.8. All the spectral data are in agreement with the structure of the **DMC** compound.

3.2. (*E*)-3-(4-methoxyphenyl)acrylic acid (MCA)

Obtained in 85% yield as white crystal; m.p. 170ºC. UV: λ_{max} (EtOH) 228 dan 308 nm. Spectrum IR (KBr; υ cm^{-1}): 2936, 2844, 2561, 1685, 1623, 1598, 1255, 1173, 827 cm^{-1} . ^{1}H-NMR (DMSO) 3.78 (3H, s), 6.60 (1H, d, J = 16Hz), 6.96 (2H, d, J = 5.0 Hz), 7.53 (1H, d, *J* = 16 Hz), 7.62 (2H, d, *J* = 5.0 Hz). ^{13}C-NMR (DMSO-d6, δ ppm) : 55.5, 114.5, 116.7, 127.0, 130.1, 143.9, 161.1, 168.0. All the spectral data are in agreement with the structure of the **MCA** compound.

3.3. (*E*)-methyl-3-(4-methoxyphenyl)acrylate (MC)

Obtained in 75% yield as white crystal; m.p. 46ºC. UV: λ_{max} (EtOH) 230 dan 314 nm. Spectrum IR (KBr; υ cm^{-1}): 3378, 1687, 1268, 1177, 1617, 823. Spectrum ^{1}H-NMR (CD_3OD; TMS; δ ppm) : 9.96 (1H,s), 6.75 ppm (2H, d, *J* = 8.2 Hz), 7.50 ppm (2H,d, *J* = 8.2 Hz), 3.65 (3H, s), 7.52 ppm (1H, d, *J*=15.8 Hz), 6.35 (1H, d, *J*=15.8 Hz.). Spectrum ^{13}C-NMR (DMSO-d6, d, ppm): 167.6, 160.4, 145.3, 130.8, 130.8, 125.6, 116.3, 116.3, 114.4, 51.7. All the spectral data are in agreement with the structure of the **MC** compound.

3.4. 3-(4-methoxyphenyl)propanoic acid (MPPA)

Obtained in 83% yield as white crystal; m.p. 96ºC. UV: λ_{max} (EtOH) 224 nm; dan 278 nm. Spectrum IR (KBr; υ cm^{-1}) : 2930, 2835, 2624, 1702, 1512, 1301, 1246, 1216, 821. Spectrum ^{1}H-NMR (CD_3OD; TMS; δ ppm): 6.88 (2H, d, *J* = 6.0 Hz), 7.15 (2H, d, *J* = 6.0 Hz), 3.82 (3H, s), 2.88 (2H, t, *J*= 6.0 Hz), 2.54 (2H, t, *J*=9.0 Hz). Spectrum ^{13}C-NMR (CD_3OD, d, ppm): 175.4, 158.7, 133.9, 130.3, 129.9, 114.9, 114.5, 55.3, 36.9, 30.9. All the spectral data are in agreement with the structure of the **MPPA** compound.

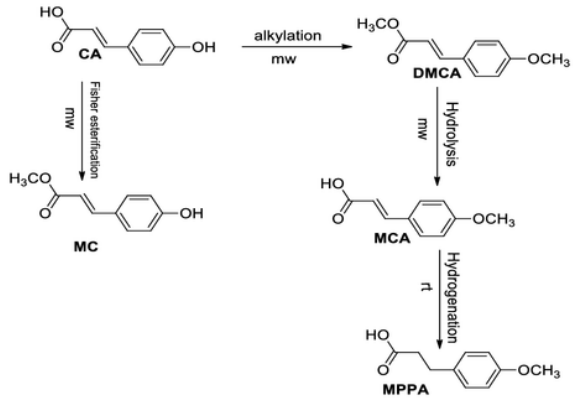


Fig 2.1. Structure Modification of *p*-coumaric acid (CA)

In this research, syntheses of DMCA, MCA, and MC were succesfully conducted by microwave irradiation. The basic mechanism of microwave iradiation is caused by the agitation of polar or ionic molecules that move because of magnetic field movement. The occurrence of these magnetic movements causes the particles to try to orientate or parallelize with the field, limiting the movement of particles due to the interaction between particles and dielectric resistence. This will cause heat that centers on the magnetic plate. Microwave iradiation is different from the conventional heating method since in the conventional heating, oil bath or heating mantle is heated first followed by its solvent. This kind of heat distribution will cause heat differences between the mantle and the solvent (11; 12).

The alyklation reaction between the phenol group of CA with dimethylsulphate was conducted according to the principle of Williamson ether synthesis (13). In this condition, there should be no water that may causes the hydrolysis of dimethyl sulphate, so acetone was used as a solvent. Baytas et al. (2012), also reported the use of dimethylsulphate on methylation reaction of the derivate of cynamate compounds namely Azragel, that has activities as antiplatelet which inhibited the formation of thromboxane (14).

Besides being able to alkylating the OH- phenolic moiety of CA, dimethyl sulphate can also be used to the alkylation of carboxylate moiety. Potassium carbonate was used to form the carboxylic ion of CA so that its nucleophilic property increased to react with methyl group of dimethyl sulphate (15).

The hydrolysis of DMC into MCA was performed in the base condition using KOH as reagent with ethanol as a solvent according to our previous research (16). This method was selected because it is an irreversible reaction with the formation of potassium *p*-methoxycoumarate salt, then was acidized with HCl to obtain MCA compound.

The structure modification from MCA to MPPA was conducted through the catalytic hydrogenation reaction with Raney-Ni as the metal catalyst. The working mechanism of Raney-Ni metal is to reduce the double bond of MCA : (i) vinilyc double bond adsorption on the surface of the hydrogenated metal catalyst, (ii) the β-carbon of the MCA attached hydrogen to form a σ- bond between the metal and α-C, and finally (iii) reductive elimination of the free alkane, MPPA (10;17).

Modification of CA structures into DMC, MCA, MC and MPPA causes changes in physicochemical properties of the molecules, including log P, MR and Etotal. Changes in

molecular properties result in differences in the nature of their interactions with target molecules (receptors) that can be expressed as rerank score (RS). The physicochemical properties of the compound are shown in Table 2. Log P describes the ratio of molecular affinity of nonpolar solvents versus polar solvents representing lipophilic character of QSAR study (18). MR is a molecular size influenced by its polarizability that classified as steric property, E_{total} is molecular free energy in the most stable conformation that is one of the electronic properties of a molecule, whereas RS is a free energy of ligand-protein binding that exhibits the affinity of the compound against a selected target molecule (19), i.e. COX-1 enzyme (PDB ID 1CQE).

3.5. Antiplatelet activities test conducted with the clotting time slide method

Low dose aspirin can be used as an antiplatelet and inhibitor of COX-1 (3; 21). Based on this fact, the anti-platelet test was performed according the aspirin doses. The results of antiplatelet of each compound group with slide clotting time method are: CMC-Na (129.7 ± 6.39); CA (253.5 ± 11.61); DMC (355.6 ± 8.79); MCA (301.8 ± 11.66); MC (263.7 ± 12.89); MPPA (211.8 ± 11.66), ASP (339.2 ± 9.9) seconds as shown in Fig.2.

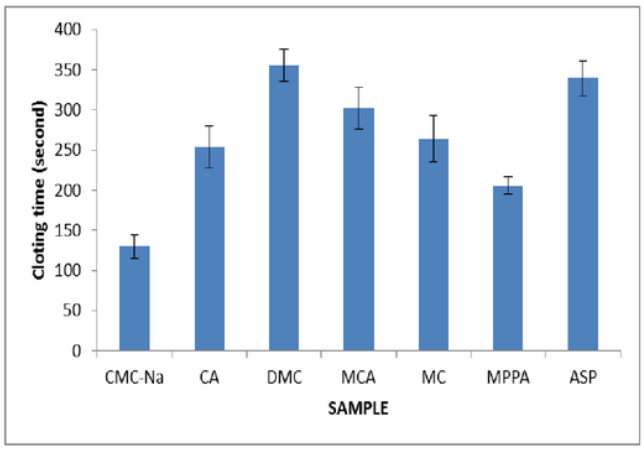


Fig 2. Histogram of antiplatelet activity **(mean ± SE)** of CMC-Na, CA, DMP, MCA, MC, MPPA, ASP by slide clotting time method

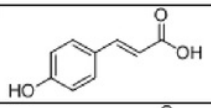
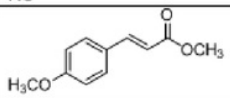
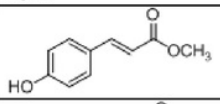
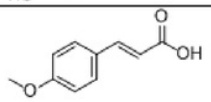
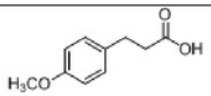
Based on the ANOVA statistical test Fig.2. it can be seen that the blood clotting time of the CA and its derivates (DMC, MCA, MC, and MPPA) have significant differences with the negative control group, namely the CMC-Na test group ($p<0.05$). This suggests that CA and its derivatives have antiplatelet effects. The DMC, MCA, and MC groups clotting times show significant improvements compared to the CA. While MPPA has significantly different clotting time compared to all test groups ($p< 0.05$), it has a lower antiplatelet effect than other derivatives. All synthesized compounds give greater effect than negative control. DMC and MCA compounds have clotting times that are not significantly different from the positive control of aspirin (ASP), which shows its potential as the same antiplatelet.

This can be explained with the *in silico* study of COX-1, where the docking score in the form of rerank scores (RS) according to the MVD 5.5 program are shown in Table 2.

The RS of ASP is -68.6619 kcal/mol. The RS values of the CA derivatives, namely DMC, MCA, and MC, are higher than CA. Meanwhile, the lowest RS MPPA compounds show similarly low interactions with COX-1 and thromboxane. The best docking poses of ASP and CA derivatives are shown in Fig. 3. The interactions of the functional groups of each test compound with the COX-1 amino acid residue is shown in Fig. 4A-F.

The COX-1 enzyme was constitutively expressed in most tissues, in which it controlled the synthesis of of prostaglandins. COX-1 is the only form of enzyme present in mature thrombocyte and is also present in the blood vessels of endothelium, gastrointestinal epithelium, brain, spinal cord, and kidney (20).

Table 2. Rerank Scores (RS), log P, MR, Etotal, of *p*-coumaric acid derivatives

Compound	Chemical Structure	RS (kcal/mol) PDB 1CQE	Log P	MR (cm^3/mol)	E_{total} (kcal/mol)
CA		-69.0016	1.54	44.67	10.499
DMC		-75.7788	2.07	55.54	13.876
MC		-73.5214	1.81	50.10	6.750
MCA		-71.8997	1.81	50.10	17.469
MPPA		-66.5918	1.56	48.25	6.4017

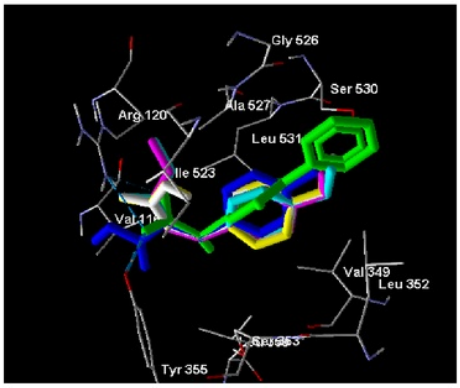


Fig 3. Docking pose FLP (green), CA (yellow), DMC (light blue), MC (pink), MCA (white), MPPA (dark blue), on cavity 5 (Vol 54.272) of COX-1 (PDB 1CQE)

In Fig. 4, (A-F) it can be seen that all molecules of the test compound are in the same bonding place as FLP_1650 [A], namely cavity-5. RS FLP is -88.6182 kcal / mol. The RS data in Table 2, which is the energy of enzyme-ligand interaction (ΔG, kcal/mol), shows the amount of energy of Van der Waals, electrostatic and hydrogen bonding interactions, and it can be used to predict how strong the ligand-enzyme bonds. The presence of methoxy groups in DMC and MCA increases the strength of interaction with enzymes, especially with Leu352 residues. The CA and all derivates having carboxylate groups form the hydrogen bond on the -COOH moiety with the amino acid residues of Arg 120 of COX-1 enzyme as in the ligand reference, nameley flubiprofen (FLP_1650 [A]) as reported by El-wahab et al. (2011) (7). The double bond change of vynilic in MCA into single bond in MPPA converts the hybridization of the rigid sp2 bond into freely rotating sp3, resulting in weaker interactions with the residue.

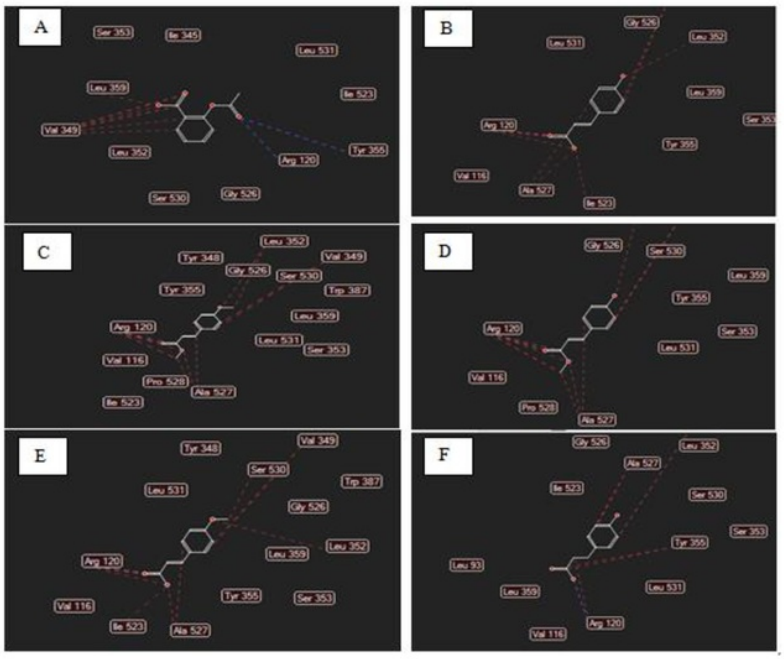


Fig 4. 2D pictures showed interaction of ASP (4A), CA (4B), DMC (4C), MC (4D), MCA (4E), and MPPA (4F) with amino acid residues in cavity 5 of enzyme COX-1 (PDB 1CQE).
Hydrogen bonding in blue dashed-line; steric interaction in red dashed-line.

As *in silico* study results, it appears that the CA molecule and its derivatives inhibit COX-1 enzyme. COX-1 binding CA and its derivatives in blood platelets will prevent COX-1 pathway to produce thromboxane-A2 (TXA2) and afterward restrain platelet aggregation for the period of platelets' lifecycle (20;.21).

3.6. The relationship between physicochemical properties and biological activity

The statistical analysis was carried out by regression method between each dependent variable against antiplatelet activity, with one independent variable of physicochemical property. The result showed a linear relationship between physicochemical properties and antiplatelet activity among 5 tested compounds. The result is supported by the

physicochemical properties, especially lipophilic (log P) and RS properties. It may occur because in the determination of antiplatelet activity, the tested compound is administered through oral route to experimental animals. So, the compounds penetrated the cell membrane barrier to the action site depend on their lipophilic property. In this case the lipophilic nature (log P) appears to indicate a significant role. There is a significant linear relationship ($P<0.05$) between Log P (partition coefficient) and antiplatelet activity (blood clotting time). The significant linear relationship ($P<0.05$) also occurs between docking score (RS) and blood clotting time (CT) as indicated by the equations :

(1) $CT = 227.749 \text{ Log P} - 123.102$ (n= 5, adjust R^2= 0.785, F= 15.163, P= 0.030)

(2) $CT = -13.228 \text{ RS} - 666.662$ (n= 5, adjust R^2= 0.713, F= 10.927, P= 0.046)

Based on these equations it is known that antiplatelet activity is related to the lipophilic nature of the compound (Log P) and its affinity (RS) with the selected target molecule, i.e COX-1 enzyme (PDB ID 1CQE). The higher the log P value, the antiplatelet activity will increase according to the coefficient of variable having a positive value. Whereas in terms of RS, the more negative the free energy of the interaction between the compound and COX-1 then the antiplatelet activity will increase.

4. Conclusion

Four derivatives of *p*-coumaric acid have been obtained through some reactions, i.e. Fisher esterification (MC), alkylation (DMC), hydrolysis (MCA), and catalytic hydrogenation (MPPA). All *p*-coumaric acid derivatives have anti-platelet activities. The presence of methyl group increases the activity, whereas the change of double bond of vinylic into single bond in MPPA decreases the interaction with COX-1, hence decreased its antiplatelet potentials compared to those of other derivatives.

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There is not any conflict of interest in this study.

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